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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Gelfand *et al.*

Application No.: 10/052,417

Group Art Unit: 1655

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Examiner: Souaya, J.

For: THERMOSTABLE DNA POLYMERASES INCORPORATING NUCLEOSIDE TRIPHOSPHATES LABELED WITH FLUORESCEIN FAMILY DYES

Attorney Docket No.: 1803-0329-999

DECLARATION UNDER 37 CFR § 1.132
OF DAVID GELFAND

Sir:

I, David Gelfand, declare that:

1. I am an inventor, along with Lisa Kalman, Fred Reichert, Christopher Sigua, and Thomas Myers, of what is claimed in U.S. Application No. 10/052,417. The Application is a continuation of U.S. Patent No. 6,346,379.

2. Since December 1991, I have been employed by Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501, where I now hold the dual positions of Vice President, Discovery Research Division and Director, Program in Core Research. Prior to my current employment, I held the positions of Vice President, Scientific Affairs and Director Core Technology, PCR Division and Senior Scientist with Cetus Corporation. I was employed by Cetus Corporation for over fifteen years. I have over 30 years post-doctorate experience in

biochemistry, genetics and molecular biology. Briefly, I received my undergraduate Biology degree from Brandeis University in 1966, and my Ph.D. from the University of California, San Diego in 1970, where I worked with Dr. Masaki Hayashi. My doctorate focused on DNA-dependent RNA-directed protein synthesis *in vitro*, particularly temporal control of transcription and translation. For my post-doctoral fellowship (1971-1972), I worked with Dr. Hayashi on a similar project. I was subsequently employed at the Department of Biochemistry and Biophysics of the University of California, San Francisco from 1972 to 1977, working in the laboratory of the late Gordon M. Tomkins. While in Dr. Tomkins' laboratory, I worked on (1) the effect of oncogenic viral transformation on the regulation of gene expression in cultured mammalian cells, (2) hormonal regulation of gene expression, (3) the isolation and characterization of mutants defective in tyrosine aminotransferase activity and (4) the construction of hybrid DNA molecules, genetic transformation and regulated expression of heterologous DNA fragments in *E. coli*. In 1976, I joined Cetus Corporation where I founded the Recombinant Molecular Research Division. I have over 45 scientific publications and I am inventor or co-inventor of 45 issued United States Patents. In fact, I was recognized with the Distinguished Inventor Award in 1990 for the invention of Taq DNA polymerase which was designated as the first "Molecule of the Year" by the journal Science (volume 246, cover and pages 1541, 1543-1544, 1989).

3. I understand that Application No. 10/052,417 claims a recombinant thermostable DNA polymerase that in its native form comprises the amino acid sequence LeuSerXaaXaaLeuXaaXaaProXaaXaaGlu (SEQ ID NO: 1). The "Xaa" at positions 3, 4, 6, 9,

and 10 of said sequence may be any amino acid residue, and "Xaa" at position 7 of said sequence is Val or Ile. Additionally, the "Xaa" at position 4 is mutated from its native residue to any other residue except Glu. I understand the recited thermostable DNA polymerase has a reduced level of discrimination against incorporation of nucleotides labeled with fluorescein family dyes relative to the native form of said polymerase. I understand that claims are also directed to a nucleic acid sequence encoding a recombinant form of the above-recited polymerase, sequencing methods, methods of producing labeled nucleic acid using the above-recited polymerase, and various kits utilizing the invention.

4. I also understand that during prosecution of the parent U.S. Patent No. 6,346,379, similar claims were rejected by the P.T.O. as allegedly not having adequate enabling support in the specification to support the claims, as the specification taught a single example of the class of mutants claimed. Though the specification taught routine methods to construct the other claimed substitution mutants, as well as standard methods to assay for the relevant activity, the P.T.O. questioned whether it would require undue experimentation for those skilled in the art to make and use the claimed invention. In order to demonstrate that the description in the specification of Application No. 10/052,417 is sufficient to enable one of average skill in the art to make and use the invention claimed in Application No. 10/052,417, we conducted the routine experiments described below.

5. I supervised routine experiments, guided by the specification of Application No. 10/052,417, which showed that a thermostable DNA polymerase with any amino acid other than glutamic acid at position number four in the "Critical Motif"

(LeuSerXaaXaaLeuXaaXaaProXaaXaaGlu) exhibits reduced discrimination against nucleotides labeled with fluorescein family dyes as compared to a polymerase with a glutamic acidic residue at position 4. The Critical Motif is described in Application No. 10/052,417.

6. The Critical Motif is instrumental in discriminating against incorporation of fluorescein family dye-labeled nucleotides into a nucleic acid by a given polymerase. This region is highly conserved in thermostable DNA polymerases. *See* Application No. 10/052,417, page 17, lines 10-24, and page 15, Table I. However, because the DNA polymerases of each thermophilic species are unique, the amino acid position of the region of criticality is distinct for each enzyme. Specifically, whereas in Application No. 10/052,417, position four of the Critical Motif is at residue 681 of the exemplary *Thermus aquaticus* (*Taq*) DNA polymerase enzyme, in the experiments described in this declaration, position four of the Critical Motif is at residue 683 of the *Thermus thermophilus* (*Tth*) DNA polymerase. *See* Application No. 10/052,417, page 15, Table I. As shown in Table I, the Critical Motif comprises amino acids L678 through E688 in the *Taq* DNA polymerase and amino acids L680 through E690 in the *Tth* DNA polymerase. Further, the Critical Motifs in the *Taq* and *Tth* DNA polymerases share 100% amino acid sequence identity (as do *Thermus* species Z05 and *caldophilus*). The *Thermus aquaticus* and *Thermus thermophilus* polymerases were each described in P.C.T. Patent Publication No. WO 92/06200, which was incorporated by reference in its entirety in Application No. 10/052,417.

7. In the experiments described herein, in addition to the single substitution mutations in the Critical Motif, there is an additional mutation from glycine to glutamic acid at

residue 46 in the 5'-nuclease domain of the DNA polymerase (G46E). Mutations at this site, in the motif A(V/T)YG, inactivate the 5'-nuclease activity of thermostable DNA polymerases. *See* U.S. Patent No. 5,466,591. This mutation serves to greatly attenuate the 5'-nuclease activity of the polymerase without affecting the polymerase domain, processivity, *etc.* The G46E mutation is present in each polymerase analyzed herein. The G46E mutation should have no effect on discrimination against incorporation of nucleotides labeled with fluorescein family dyes in E683x mutants, as the mutation is 637 residues away and in a completely separate structural domain. In addition, the activities of all of the mutant DNA polymerases described herein are compared to the activity of a parent polymerase with a wild-type polymerase domain and the G46E mutation in the 5'-nuclease domain. Thus, the altered activities of the mutant polymerases relative to the parent polymerase result from the mutations in the Critical Motif, not the G46E mutation.

8. The 19 possible G46E E683x *Tth* substitution mutants were constructed using routine recombinant DNA techniques described in Application No. 10/052,417. *See* the specification at page 17, line 25 - page 18, line 20. Briefly, 19 different double stranded DNA segments encoding *Tth* DNA polymerase amino acids 679 through 702 (encompassing the *Tth* polymerase Critical Motif, amino acids L680 through E690) with a single mutation at E683 were substituted for the wild-type amino acid-encoding sequence in a *Tth* DNA polymerase expression vector. The expression vector was analogous to pLSG32. *See* U.S. Patent No. 5,618,711. Two vector-unique restriction enzyme sites were used to facilitate the substitutions. The intended amino acid sequence was confirmed for each clone by DNA sequence analysis. Following induced expression and enzyme purification as taught by Application No. 10/052,417 (*see* the

specification at page 19, lines 3-14, and page 34, line 20 - page 35, line 10), we easily obtained the 19 alternate E683x variants of *Tth* DNA polymerase.

9. DNA polymerases mutated at position four of the Critical Motif to a residue other than glutamate exhibit reduced discrimination against nucleotides labeled with a fluorescein family dye. To evaluate the ability of E683x mutants to incorporate nucleotides labeled with a fluorescein family dye relative to the E683 polymerase, a routine competition assay was conducted. The competition assay was analogous to the assay described in Example II of Application No. 10/052,417 and gives comparable results.

10. In the competition assay, the G46E E683 *Tth* and all 19 possible G46E E683x *Tth* substitution mutant DNA polymerase enzymes were evaluated for their relative ability to incorporate unlabeled dCMP versus a fluorescein dye-family labeled analog, HEX-2-PA-dCMP. Each enzyme was analyzed in a competition assay for the incorporation of [α-^{33}P]-dCMP, with the competitors being either unlabeled dCTP or HEX-2-PA-dCTP. The reaction mixture comprised template DNA, dATP, dGTP, dTTP, and [α-^{33}P]-dCTP with unlabeled dCTP. Competition reactions comprised the reaction mixture, a G46E E683 *Tth* or G46E E683x *Tth* mutant polymerase at limiting enzyme concentrations, and either unlabeled dCTP or HEX-2-PA-dCTP at varying concentrations. The titration range was adjusted relative to the ability of each enzyme to incorporate HEX-2-PA-dCTP. Control reactions for the competitor substituted water for the competitor. Background from the [α-^{33}P]-dCTP was determined by substituting EDTA for the competitor to bind all divalent magnesium, thereby precluding any DNA polymerase activity. After mixing, the competition reactions were covered and incubated

for ten minutes at 75 degrees Celsius. After the reaction, the DNA was precipitated, cleaned, and quantified by liquid scintillation spectrometry. For each titration point, the picomoles (pmol) of incorporated [α-^{33}P]-dCMP were determined. These data were graphed. An equation derived from the best-fit line was used to calculate the concentration of competitor required to reduce [α-^{33}P]-dCMP incorporation by 50%. Data from the competition assay is shown in Figure 1.

11. Enzymes that incorporate HEX-2-PA-dCMP poorly require relatively higher concentrations of HEX-2-PA-dCTP to reduce the amount of [α-^{33}P]-dCMP incorporated by 50% as compared to the unlabeled dCTP control. In contrast, enzymes that discriminate less, or are improved in their ability to incorporate the fluorescent analog, require relatively lower concentrations of HEX-2-PA-dCTP to reduce the amount of [α-^{33}P]-dCMP incorporated by 50%.

12. These data show that G46E E683 *Tth* DNA polymerase has 19-fold discrimination against HEX-2-PA-dCTP relative to unlabeled dCTP. In contrast, every E683x mutant enzyme shows significantly reduced discrimination against HEX-labeled nucleotides relative to the G46E E683 *Tth* DNA polymerase enzyme with a wild-type active site. That is, as described in the specification of Application No. 10/052,417, a mutation from glutamic acid (E) to any other residue results, without exception, in significantly reduced discrimination against nucleotides labeled with fluorescein family dyes.

13. Specifically, the G46E E683D *Tth* shows about a 10-fold discrimination against HEX-2-PA-dCTP (1.72-fold, *i.e.*, 172% improved). The G46E E683N *Tth*, G46E E683Q *Tth*, G46E E683S *Tth*, and G46E E683T *Tth* mutant enzymes show a 2- to 4-fold discrimination

against HEX-2-PA-dCTP (almost 5-fold to 10-fold, *i.e.*, 500% to 1,000% improved). The remaining G46E E683x *Tth* DNA polymerase mutant enzymes (where x = A, C, F, G, H, I, K, L, M, P, R, V, W, Y) show a less than 2-fold discrimination against HEX-2-PA-dCTP, *i.e.*, greater than a 10-fold (1,000%) improvement over the G46E E683 *Tth* enzyme. In particular, the E683F, E683G, E683K, E683L, E683M, E683P, E683R and E683W mutant enzymes incorporate the fluorescein dye-family labeled HEX-2-PA-dCTP analog better than unlabeled dCTP.

14. Mutations E683K, E683R, and E683H were described to be especially effective in incorporating nucleotides labeled with fluorescein family dyes in the specification of Application No. 10/052,417. *See* the specification at page 11, lines 8-13 and lines 17-26 for corresponding *Taq* polymerase mutants. These mutants exhibit a greater than 10-fold (1,000%) improvement over a polymerase that is not mutagenized at residue 683. Significantly, the E683R mutant enzyme is the most improved for incorporation of the fluorescently labeled dNTP analogs. This substitution mutant enzyme was described as most preferred, indicating that it is especially effective in incorporation of nucleotides labeled with fluorescein family dyes. *See* the specification at page 12, lines 14-23 for a similar corresponding *Taq* polymerase mutant.

15. A direct incorporation extension assay was performed to verify that results from the competition assays are due to competition from (*i.e.*, incorporation of) HEX-2-PA-dCMP, and not merely an inhibitory effect of the HEX-labeled nucleotide.

16. The direct incorporation extension assay was limited to four enzymes: the G46E

E683 *Tth* DNA polymerase (*Tth* pol); the mutant enzyme with the highest incorporation level, G46E E683R *Tth* pol; and two mutant enzymes with intermediate phenotypes, G46E E683K *Tth* pol and G46E E683Y *Tth* pol. The extension assay was composed of pre-primed M13mp18 template DNA, an excess of DNA polymerase, all four dNTPs, and either 0, 1 or 4 μM of HEX-2-PA-dCTP. After the reaction, a purification step removed excess unincorporated dNTPs and HEX-2-PA-dCTP. The amount of HEX-2-PA-dCMP incorporated was directly determined by fluorescence. The result was normalized to the DNA extension product by UV absorption measured at 260 nm. Data from the direct incorporation assay is shown in Figure 2.

17. These data show that the three mutant *Tth* pol enzymes incorporate HEX-2-PA-dCMP much more effectively than G46E E683 *Tth* pol. The G46E E683 *Tth* pol incorporates HEX-2-PA-dCMP at barely detectable levels, only slightly above background. In contrast, the HEX fluorescence signal is significantly over background for the E683K, E683Y, and E683R mutant *Tth* pol reactions. The mutant *Tth* pols show an improvement relative to G46E *Tth* pol at the 1 and 4 μM HEX-2-PA-dCTP conditions of at least 15-fold for E683K *Tth* pol, 28-fold for E683Y *Tth* pol, and 51-fold for E683R *Tth* pol. Significantly, all four *Tth* pols exhibit approximately a four-fold increase in HEX fluorescence signal as the HEX-2-PA-dCTP concentration increases from 1 to 4 μM, indicating a direct correlation between HEX-2-PA-dCTP concentration and incorporation.

18. The results from the direct incorporation assay confirm that the results from the competition assay are due to competition from the fluorescein family dye-labeled nucleotide, rather than an inhibitory effect of the fluorescein family dye-labeled nucleotide.

19. In the specification of Application No. 10/052,417, it was described that "the specific change of E to K is not as critical to the invention as is the now identified position within the region of criticality." Page 11, lines 15-17. The specification in Application No. 10/052,417 taught the routine methods used to construct all 19 possible mutants substituted at position four. Using sequence analysis methodologies well known in the art, we discovered that a mutation in position four of the Critical Motif in a DNA polymerase to any amino acid other than glutamic acid would lead to reduced discrimination against incorporation of nucleotides labeled with fluorescein family dyes, as described in Application No. 10/052,417. We carried out a modified form of the competition assay described in Example II of the specification of Application No. 10/052,417, as described in paragraphs 10-13, above, to confirm this discovery. Essentially, the competitive incorporation DNA synthesis assay asks how much better or worse a particular mutant enzyme incorporates the fluorescein dye family labeled analog nucleotide as compared to the synonymous "normal" nucleotide. DNA synthesis reactions are routinely performed in the art. The results from the experiments described herein, conducted according to the methods described in Application No. 10/052,417, confirmed the description in the Application perfectly.

20. As demonstrated by the experiments described above, one of ordinary skill in the art could make and use the recited polymerases using only routine experimentation guided by the specification of Application No. 10/052,417.

21. I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing therefrom.

David H. Gelfand

David H. Gelfand, Ph.D.

12-20-02

Date